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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Although the basic underlying mechanisms of Parkinson's disease remain unknown, considerable efforts have centered on developing effective strategies for halting the neurodegenerative process and restoring normal function. One promising approach involves the potential therapeutic use of neurotrophic factors. The main hypothesis being tested in this research project is that the neuregulin glial growth factor-2 (GGF2), a neural growth/differentiation factor, is neuroprotective and/or neurorestorative for the damaged dopaminergic nigrostriatal system. Our present results provide good evidence that GGF2 can function as a neurotrophic factor for nigrostriatal dopaminergic neurons. Studies in vivo demonstrate that administration of GGF2 to the normal nigrostriatal system enhances striatal dopamine release. This is important because compounds that can stimulate the secretion or release of dopamine in the nigrostriatal system have the potential for overcoming the lack of dopamine neuronal function in Parkinson's disease patients. Results from in vitro experiments show that GGF2 protects cultured midbrain neurons against injury, most notably neurotoxin-induced degeneration. In addition, treatment of the cultures with GGF2 promotes the long-term survival of the developing dopamine cells. Thus, GGF2 exhibits trophic actions for mesencephalic dopamine neurons, which may be mediated in part via glial mechanisms. Overall, results from these studies may form the basis for the therapeutic application of neuregulins to the treatment of neurotoxin-induced neurodegenerative disorders such as Parkinson's disease.

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INTRODUCTION

The main hypothesis being tested in this research project is that the neuregulin glial growth factor-2 (GGF2) is neuroprotective and/or neurorestorative for the damaged dopaminergic nigrostriatal system. Other hypotheses to be tested are that GGF2 augments functional indices of the dopaminergic nigrostriatal system and that one of the intracellular signaling mechanisms mediating the protective effects of neuregulins for dopamine neurons invloves the PI3-kinase pathway. These studies are being conducted in normal rats, in a 6-OHDA rat model of Parkinson's disease, and in primary neuronal cultures of midbrain dopamine cells. Based on our preliminary findings in vivo, we expect that GGF2 will enhance several measures of dopamine neurochemistry in the intact nigrostriatal system, and protect midbrain dopamine neurons from neurotoxin-induced degeneration. Based on initial in vitro experiments, we expect GGF2 to serve as neuroprotective and differentiation factors for nigral dopamine cells. Overall, results from these studies may form the basis for the therapeutic application of neuregulins to the treatment of neurotoxin-induced neurodegenerative disorders such as Parkinson's disease.

BODY

Experiment #1a: This experiment is the first in a series to determine if infusion of GGF2 augments nigrostriatal dopamine function in the rat. In vivo microdialysis was used to examine the acute effects of GGF2 on *basal* dopamine release in the nigrostriatal system. Our preliminary studies had indicated that unilateral infusion of the neuregulin heregulin-β1 (HRG-β1; 10 μg) just above the substantia nigra resulted in a rapid increase in dopamine overflow in the ipsilateral striatum. In the present experiment, we tested the effects of the more potent neuregulin GGF2 at a lower dose (3 μg) and, as expected, found that a single unilateral supranigral injection of GGF2 induced a rapid increase in dopamine overflow in the ipsilateral striatum. Preliminary analysis of the data (at least 6 experimental and control (vehicle-injected) animals each have been processed thus far) indicates that the GGF2-induced release of striatal dopamine is rapid (with an increase as early as 25 minutes after GGF2 injection) and substantial (at least a 200% peak increase in striatal dopamine overflow compared to baseline levels at 50 minutes after injection). By 100 minutes post-injection, the striatal dopamine concentration had returned to baseline levels. Thus, acute administration of GGF2 can enhance at least one important index of nigrostriatal function. After processing a few more rats for these basal dopamine release studies, in Year 2 we will extend these experiments to encompass the acute effects of GGF2 on *evoked* release of dopamine in the nigrostriatal system using d-amphetamine (voltage-independent overflow) or high K^+ (voltage-dependent overflow).

Experiment #2a: This neuroprotection study is designed to test if supranigral administration of GGF2 prior to neurotoxic (6-hydroxydopamine; 6-OHDA) lesion of the nigrostriatal system protects the nigral dopamine neurons (and their associated behavioral and neurochemical indices) from the neurotoxic damage. Despite successful pilot studies, we obtained inconsistent results upon processing larger groups of animals. We have now determined that the 6-OHDA lesions, themselves, targeted to the ascending medial forebrain bundle (MFB), resulted in inconsistent lesions of the nigrostriatal system as evidenced particularly by variable rotational behavior and dopamine neurochemical measures. We have since tested several alternative injections sites and doses for the 6-OHDA (e.g. supranigral, striatal, different sites in MFB) and have recently determined that the "2-site preterminal lesions" of Bjorklund and colleagues (Kirik et al., 1998) are optimal in our hands for producing robust lesions as determined by rotational behavior measures, and almost complete ("partial") lesions of the nigral dopaminergic cells, as determined by in situ hybridization and immunocytochemistry for tyrosine hydroxylase (TH). We feel these "partial" lesions, which would better mimic the situation in Parkinson's disease, are best for this and especially the subsequent neural repair study, in which the GGF2 is administered after the neurotoxin lesion. Thus, we have recently processed a large group of rats (n=32) for the pretreatment neuroprotection study using the new 6-OHDA injection site coordinates and dose; rotational behavior analyses will soon begin and continue weekly for an additional 4 weeks, after which the rats will be sacrificed and processed for either TH immunocytochemistry, TH mRNA in situ hybridization or dopamine neurochemical analysis. In the meantime, the neural repair experiments planned for Year 2, which involve post-treatment of the nigral dopamine neurons with GGF2 after the 6-OHDA-induced nigrostriatal lesions, will commence.

Experiment #3a: Probably the most successful and fruitful studies to date for this research project involve these in vitro experiments, which involve testing the effects of neuregulins on dopamine neurons in primary mesencephalic cultures upon treatment with neurodegenerative agents. Although we originally proposed to employ three different neurotoxic agents (6-OHDA, rotenone and Fe^{+}), because of the grant reviewers' comments that "The tissue culture studies are somewhat unfocussed" and "...the justification for analyzing protective mechanisms against Fe and rotenone in vitro are not clear" we decided to focus our efforts on 6-OHDA. Our results indicate that treatment with GGF2 significantly protects TH^{+} neurons (dopaminergic neurons) in the cultures against 6-OHDA-induced degeneration (see Figs 1 and 2). In addition, GGF2 also protected the dopamine cells against serum withdrawal, a somewhat milder insult and standard assay in similar cultures (see Fig. 3). To extend and complement these findings, the effects of GGF2 on the differentiation of the TH^{+} cells in these same experiments are being analyzed using dopamine uptake assays and by measuring process outgrowth in contol vs. GGF2-treated cultures.

Although originally slated for Year 2, we found it more feasible to conduct some the survival studies in Year 1. Our data indicate that GGF2 is not a short-term survival factor for dopamine neurons in these developing cultures (see Fig. 4). However, interestingly, GGF2 does appear to promote the long-term survival of the developing dopamine cells (in cultures are taken out to 21 days) (Zhang et al. 2002; see abstract in Appendix, panel 4).

In accordance with the grant reviewers' suggestion "It seems also worthwhile to assess erbB expression by embryonic neurons in culture...", we have shown that ErbB4 mRNA is expressed in rat embryonic day 14 ventral mesencephalic flexure (from which thew cultures are derived) in an overlapping pattern with TH mRNA (see panel 1 in Zhang et al. abstract). Moreover, we determined that ErbB2, ErbB3 and ErbB4 receptor proteins are all expressed in the cultures (see panel 2 in Zhang et al. abstract) and are upregulated by GGF2 treatment (panel 3 in Zhang et al. abstract).

To begin to address the possible mechanism of action of GGF2 in the midbrain cultures, we have initially assessed the role of glia in the cultures. We found that GGF2 promotes astrocytic proliferation in the developing midbrain cultures, as determined by immunocytochemistry for glial fibrillary acidic protein (GFAP), and that GFAP protein levels (as determined by Western blots) are increased in GGF2-treated compared to control cultures (see panels 5 and 6 in Zhang et al. abstract). These data raise the possibility that some of the long-term survival and neuroprotective effects of GGF2 on dopaminergic neurons in the midbrain cultures may be mediated via glia.

KEY RESEARCH ACCOMPLISHMENTS

- The neuregulin GGF2 enhances basal release of dopamine in the rat nigrostriatal system in vivo.

- GGF2 protects cultured primary dopaminergic neurons against serum deprivation and 6-OHDA-induced degeneration.

- GGF2 promotes the long-term (but not short-term) survival of dopaminergic neurons in rat primary midbrain cultures.

- The neuregulin receptors ErbB2-4 are expressed in rat primary midbrain cultures and are upregulated by GGF2 treatment.

- GGF2 promotes the proliferation of glia in primary midbrain cultures.

REPORTABLE OUTCOMES

Abstract:

Zhang, L., Fletcher-Turner, A., Lundgren, K.H., Cheng, L., Marchionni, M.A., Yurek, D.M. and Seroogy, K.B. (2002) The long-term survival of cultured dopaminergic neurons is promoted by the neuregulin GGF2. *Soc. Neurosci. Abstr.* 28: #427.6.

Presentation:

"Neuroprotection of Dopamine Cells by Neuregulins in Models of Parkinson's Disease"
(Invited Seminar presentation to Dept. of Neurology, University of Cincinnati, April 15, 2002)

CONCLUSIONS

Results from experiments performed during Year 1 of the present grant provide good evidence that the neuregulin GGF2 can function as a neurotrophic factor for nigrostriatal dopaminergic neurons. Studies in vivo demonstrated that administration of GGF2 to the normal nigrostriatal system enhances striatal dopamine release. This is important because compounds that can stimulate the secretion or release of dopamine in the nigrostriatal system have the potential for overcoming the lack of dopamine neuronal function in Parkinson's disease patients. In other in vivo studies, changes have been made in the site and dose of 6-OHDA lesioning to better address the GGF2 neuroprotection experiments. Rersults from in vitro experiments show that GGF2 protects cultured midbrain neurons against injury, most notably neurotoxin-induced degeneration. In addition, treatment of the cultures with GGF2 promotes the long-term survival of the developing dopamine cells. Thus, GGF2 exhibits trophic actions for mesencephalic dopamine neurons, which may be mediated in part via glial mechanisms.

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Kirik, D., Rosenblad, C. and Björklund, A. (1998) Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp. Neurol.* 152: 259-277.

Zhang, L., Fletcher-Turner, A., Lundgren, K.H., Cheng, L., Marchionni, M.A., Yurek, D.M. and Seroogy, K.B. (2002) The long-term survival of cultured dopaminergic neurons is promoted by the neuregulin GGF2. *Soc. Neurosci. Abstr.* 28: #427.6.

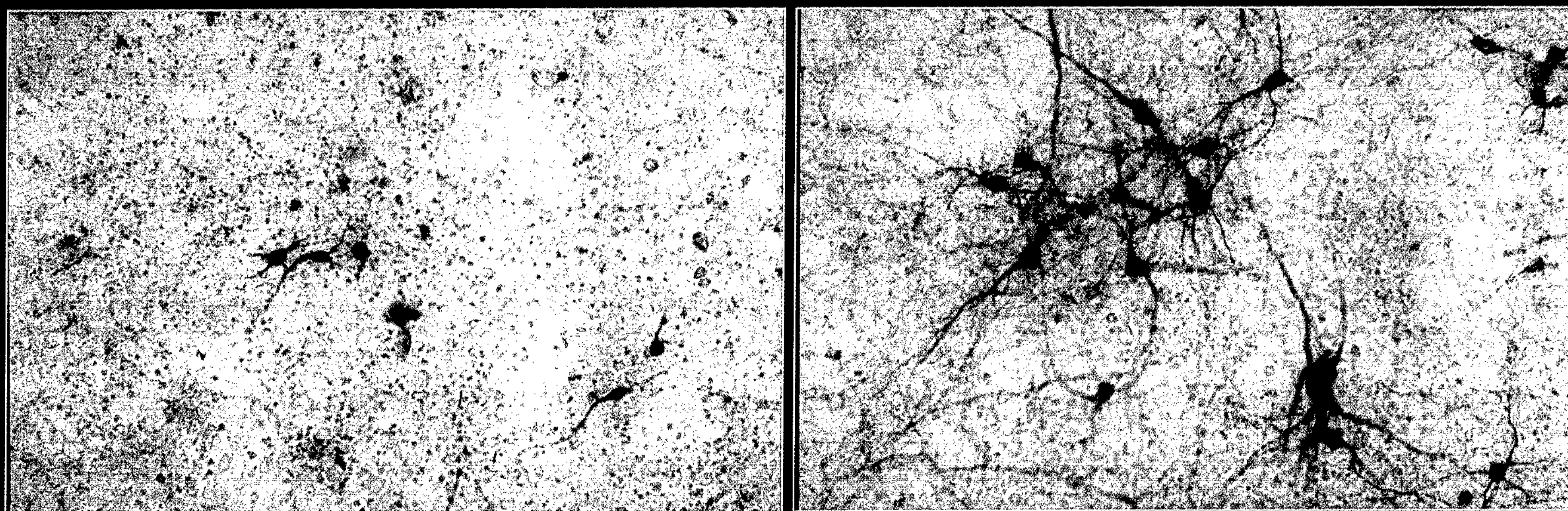
APPENDICES

Zhang, L., Fletcher-Turner, A., Lundgren, K.H., Cheng, L., Marchionni, M.A., Yurek, D.M. and Seroogy, K.B. (2002) The long-term survival of cultured dopaminergic neurons is promoted by the neuregulin GGF2. *Soc. Neurosci. Abstr.* 28: #427.6.

Yurek, D.M. and Seroogy, K.B. (2001) Neurotrophic factor protection of dopaminergic neurons. In: Neurobiology of the Neurotrophins, I. Mocchetti (ed.), F.P. Graham Publishing, New York, pp. 355-397.

Fig.1

6-OHDA-induced Degeneration

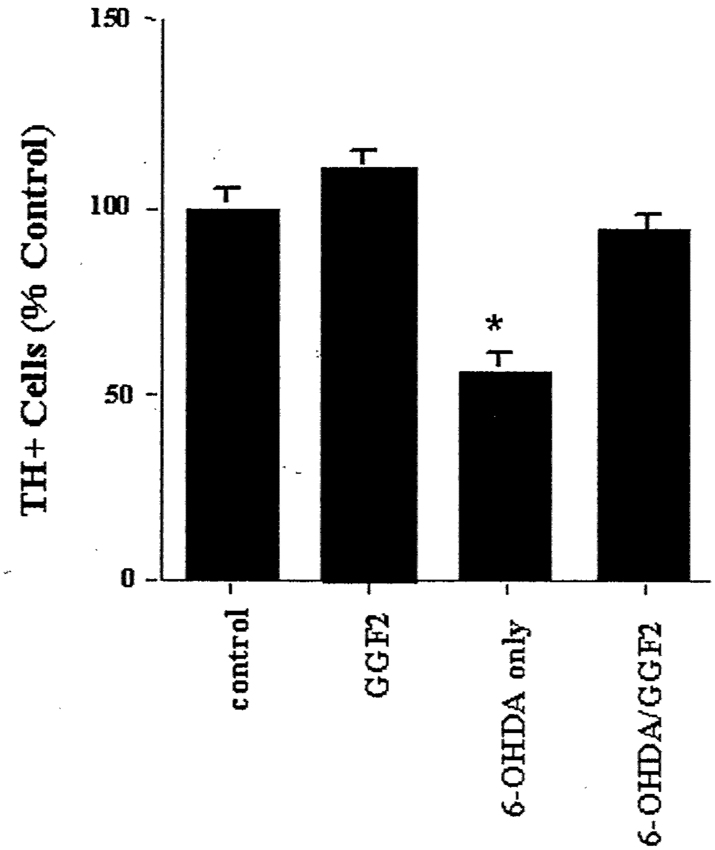


6-OHDA **6-OHDA + GGF2**

GGF-2 treatment (right panel) protects the TH+ neurons from 6-OHDA-induced degeneration in ventral midbrain cultures.

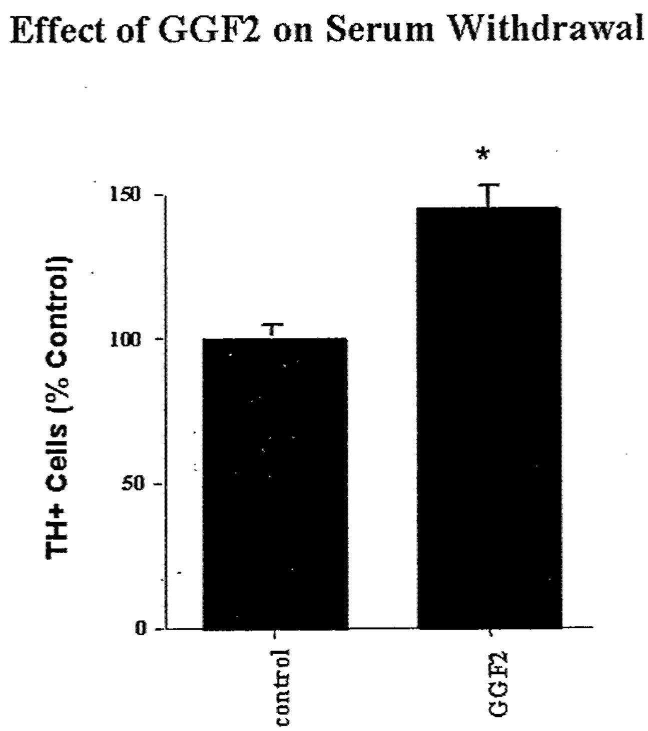
Fig. 2

GGF2 Prevents 6-OHDA-Induced Degeneration of Cultured Midbrain TH Cells



6-OHDA-induced degeneration: **Cultures were maintained in DMEM/F12/N2 medium, and treated with GGF2 (100 ng/ml) at 1 hour after plating. On DIV 4, cultures were exposed to 6-OHDA (50μM for 90 min). On DIV 9, the cultures were fixed and the number of dopamine neurons was analyzed using TH immunocytochemistry. As the graph shows above, GGF2 treatment significantly protected the TH+ neurons from 6-OHDA-induced degeneration.**

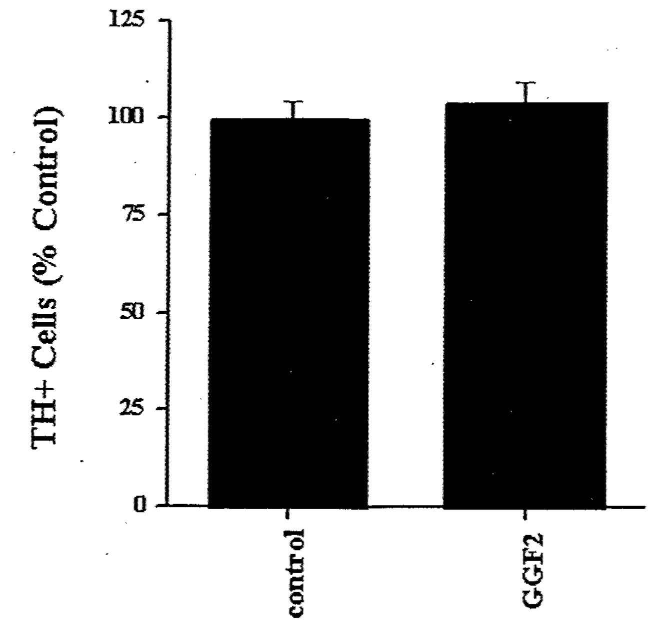
Fig. 3



Serum deprivation: **As show above, GGF2 significantly increased the number of TH+ neurons compared to the control cultures. Cultures were maintained in hormone-supplemented serum-free (HSSF) medium, which is a well-defined serum-free medium, and were treated with GGF2 (100 ng) at 1 hour after plating. Parallel cultures were treated with vehicle as control. After DIV 7, cells were fixed with 4% paraformaldehyde and processed for TH immunostaining.**

Fig. 4

Effect of GGF2 on Normal TH Cell Survival



__Survival:__ **The __short-term__ effect of GGF2 treatment on normal developing dopaminergic neurons was examined in mesencephalic cultures maintained in Dulbecco's Modified Eagle Medium and Ham's F12 nutrient mixture, supplemented with N2 (DMEM/F12/N2) medium. Cultures were treated with GGF2 (100 ng/ml) at 1 hour after plating. In control cultures, cells were treated with vehicle. On DIV 7, the TH-positive neurons were analyzed by immunostaining for TH. As shown above, no significant difference in TH+ cell numbers was found between treatment and control cultures.**

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Neurotrophic Factor Protection of Dopaminergic Neurons

David M. Yurek and Kim B. Seroogy

INTRODUCTION

While the focus of this chapter will be the protection of dopaminergic neurons by neurotrophins, we would be remiss if we did not mention how other neurotrophic factors provide support for dopaminergic neurons. Many studies have reported that individual factors provide neurotrophic support for cultured dopaminergic neurons, however, neurotrophic activity *in vivo* may require the concerted action of several or even a multitude of neurotrophic factors. Therefore, this chapter will also review recent developments for neurotrophic factors outside of the neurotrophin family that are also known to provide neurotrophic support to dopaminergic neurons both *in vitro* and *in vivo*. The important role that neurotrophic factors play in sculpting the development of the peripheral nervous system (PNS) has been well documented and it is generally accepted that these same factors play a similar role in the development of the central nervous system (CNS). Damage to nervous tissue in both the PNS and CNS typically elicit a concomitant increase in neurotrophic activity, and this suggests that neurotrophic factors are important components in the repair process of damaged neural structures *(123)*. Likewise, these same neurotrophic factors may play a therapeutic role in terms of restoring or sustaining neural function in chronic neurodegenerative diseases (*e.g.*, Parkinson's disease), acute nervous system injury, or other age-related neurodegenerative disorders. Neurotrophic factors and their receptors are dynamically expressed in central dopaminergic neurons as well as within the nigrostriatal system during development and throughout adulthood. In this chapter, we will review past and recent developments in neurotrophic factor research that is related to the ability of these molecules to promote the survival, maintenance, and protection of dopaminergic neurons.

2. NEUROTROPHINS

The neurotrophins belong to a family of related neurotrophic factors that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). BDNF is a 13.5 kDa basic protein with a completely conserved amino acid sequence among mammals *(12,149)*. All neurotrophins bind with similar affinity to the low affinity NGF receptor, p75. BDNF and NT-4/5 bind with high affinity to

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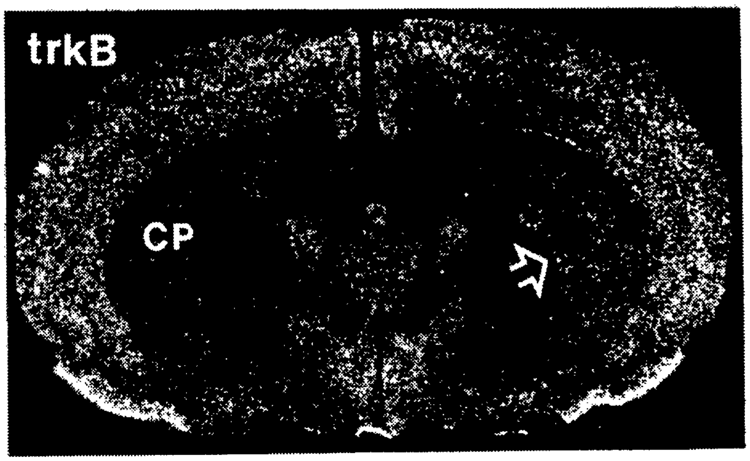

Fig. 1. Expression of trkB mRNA is increased in rat caudate-putamen (CP) (arrow) ipsilateral to a 6-OHDA lesion of the nigrostriatal pathway (right side).

the tyrosine kinase receptor, trkB. NT-3 binds with high affinity to another related tyrosine kinase receptor, trkC, and with low affinity to trkB *(171)*. Of the four neurotrophins mentioned above, only NGF has been shown to have minimal or no neurotrophic effect on dopaminergic neurons *(41,89)*.

2.1. Developmental Expression of Neurotrophins and Neurotrophin Receptors in the Nigrostriatal Pathway

The developmental expression of neurotrophic factors and their receptors may provide important clues for understanding the mechanisms by which neurotrophic factors are able to influence the survival, growth, and maintenance of neurons, and ultimately how these factors can be used therapeutically to halt the progression of neurodegeneration. Therefore, this part of the chapter is devoted to a short review of developmental expression of neurotrophin and neurotrophin receptors.

During normal development BDNF mRNA expression in the rat ventral mesencephalon peaks by approximately 2 weeks after birth and then declines to lower levels in the adult *(58,129,132)*. Similarly, NT-3 mRNA exhibits a developmental decline in the ventral midbrain, with high levels of expression present neonatally and lower levels in the adult (Fig. 3) *(129,132)*. Most, but not all, of the BDNF mRNA-and NT-3 mRNA-containing cells of the ventral midbrain are dopaminergic *(160,161)*. The neurotrophin receptors trkB and trkC are also expressed in numerous cells of the ventral mesencephalon neonatally, but no developmental changes in their mRNA expression are detected *(129,132)*. Virtually all mesencephal-

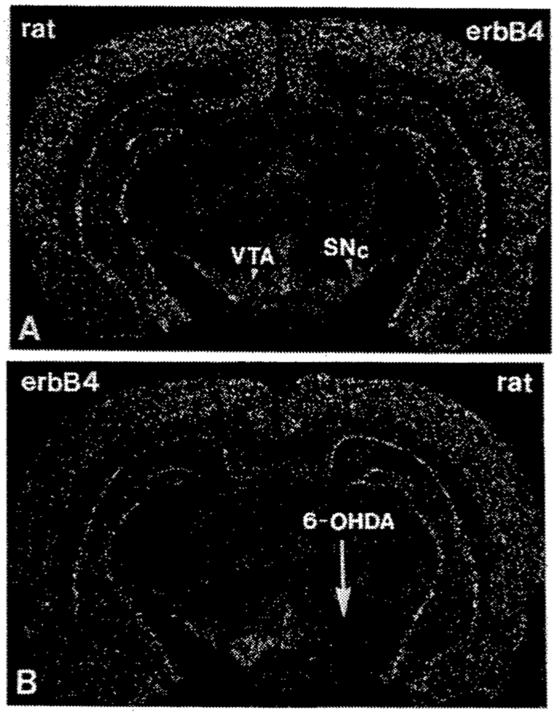

Fig. 2. Prints of film autoradiograms showing the localization of the neuregulin receptor erbB4 mRNA to the ventral mesencephalon in **(A)** normal and **(B)** 6-OHDA lesioned adult rats. **(A)** Note prominent expression of erbB4 mRNA within dopamine-containing nuclei, including the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). **(B)** Expression of erbB4 mRNA is substantially reduced in the ventral mesencephalon (arrow) ipsilateral to a 6-OHDA lesion of the nigrostriatal pathway (right side).

ic dopaminergic neurons synthesize the trkB and trkC neurotrophin receptors *(131)*. The prominent expression of both the neurotrophins and their functional receptors in the midbrain in neonatal periods suggests autocrine or paracrine mechanisms of trophic support for dopaminergic cells in early postnatal developmental events, including differentiation, maturation and target innervation. The sustained expression of BDNF and NT-3, although at lower levels, in the adult ventral midbrain suggests a role for these neurotrophins in the maintenance or repair of the mature nigrostriatal system.

Both BDNF and NT-3 mRNAs are not normally present in the adult striatum, although mRNA expression can be induced in some striatal cells following seizures *(155)*. BDNF protein, on the other hand, shows a steady increase within the striatum during the first postnatal month and is highly correlated with an increase in striatal dopamine *(210)*. Throughout the brain the trkB receptor exists in two forms: a full-length form and a truncated form, in

which the catalytic domain is absent *(122)*. In the striatum, full-length trkB mRNA increases from embryonic day 18 (E18) and reaches adult levels by birth, whereas truncated trkB mRNA is not detectable until postnatal days 10-15 (P10-15) and reaches adult levels by P30 *(60)*. During development trkB mRNA expression in the rat striatum shows a decline from postnatal days 0 to 7, which then stabilizes and remains fairly constant throughout adulthood *(116)*. Protein levels of full-length trkB in the striatum reach adult levels by E20, whereas protein levels of truncated trkB show gradual increases throughout early and late postnatal development *(60)*.

2.2. Neurotrophins: In vitro Studies

The neurotrophic and neuroprotective effects of the neurotrophins on dopaminergic neurons *in vitro* will be reviewed in this section. For culture studies, a general ranking of the neurotrophins in the order of their efficacy to promote the survival and growth of dopaminergic neurons is as follows: NT-4/5>BDNF>NT-3>NGF. The effects of individual neurotrophins on cultured dopaminergic neurons are discussed below.

2.2.1. BDNF

Addition of BDNF to dissociated mesencephalic cell cultures increases the survival of dopamine neurons, dopamine uptake, and dopamine content *(78,79, 90,218)*. BDNF added to cultures of human fetal ventral mesencephalon increased the survival, cell body size, and neuritic length of dopaminergic neurons *(172,180,218)*, and can be used to extend the storage time of free-floating roller tube cultures of fetal rat nigral tissue *(73)*. BDNF completely blocks the neurotoxic effects of 6-hydroxyDOPA on cultured dopamine neurons *(170)*. Conditioned media obtained from fibroblasts genetically modified to express BDNF improve the survival of tyrosine hydroxylase immunoreactive cells in ventral mesencephalic cultures *(85)*. Blöchl and Sirrenberg demonstrated that the addition of BDNF or NT-3, but not NGF, to mesencephalic neuronal cultures enhanced K^+-evoked and basal dopamine release that could be blocked by the tyrosine kinase inhibitors K252a or K252b *(20)*. BDNF binds with high affinity to the tyrosine kinase receptor, trkB, and these data suggest that BDNF stimulates dopamine release by binding to the trkB receptor. Binding of BDNF to trkB results in receptor dimerization, tyrosine phosphorylation, and the induction of tyrosine kinase activity. Intracellular signaling may involve the phospholipase C-γ1 and/or the c-ras pathways. However, the complete transduction sequence by which the binding of BDNF to trkB signals the release of dopamine has yet to be determined *(150)*. NGF has been shown to stimulate dopamine release from cultured dopamine neurons, but only at very high doses and its affect on dopamine release appears to be mediated by the activation of the p75 receptor because it

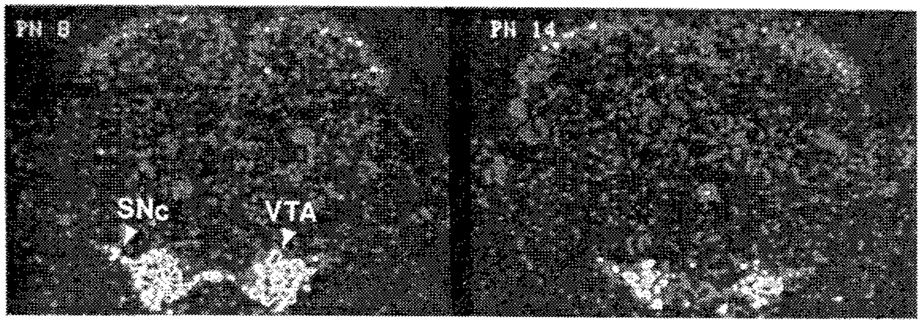

Fig. 3. Pseudocolor images of film autoradiograms showing the developmental decrement in expression of NT-3 mRNA in rat ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) from postnatal day (PN) 8 to 14. Similar results were obtained for BDNF mRNA expression in adjacent sections. (From collaborative studies of K. Seroogy and C. Gall.)

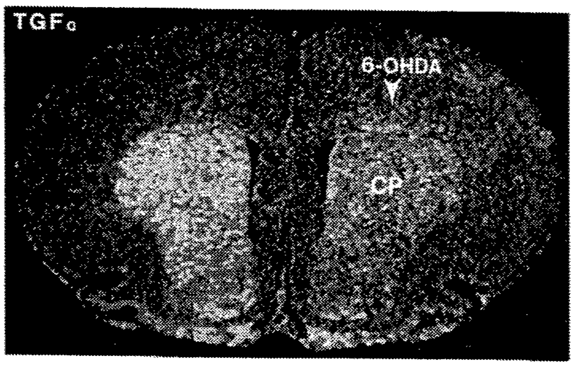

Fig. 4. Pseudocolor densitometric image showing the decreased expression of TGF-α mRNA in adult rat striatum (caudate-putamen, CP) ipsilateral to a 6-OHDA lesion of the nigrostriatal pathway (right side).

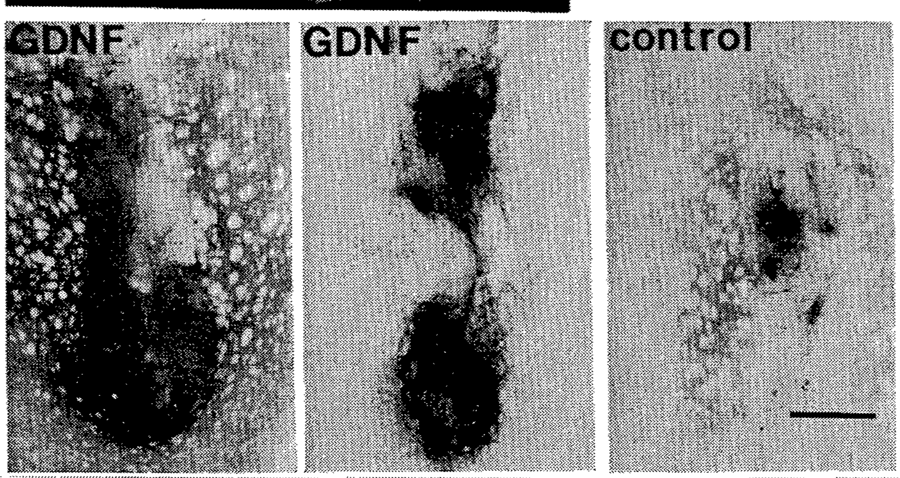

Fig. 5. Intrastriatal transplants of embryonic dopaminergic tissue treated with GDNF. Brain sections were immunocytochemically stained for tyrosine hydroxylase (TH) (brown), a marker for dopaminergic neurons. Transplants were implanted into denervated striatum and infused with GDNF or vehicle alone (control). GDNF-treated transplants typically contained 3-4 times as many TH-stained neurons than transplants that did not receive GDNF treatment (control). Scale bar = 500 μm. This figure is reproduced with permission from Academic Press.

can be blocked by the p75 antibody, MC192, but not by tyrosine kinase inhibitors *(20)*. In addition to its effects on dopaminergic neurons, BDNF can also be used to induce cortical neurons to express a dopaminergic phenotype. For example, adding BDNF plus dopamine [or dopamine agonists] to cultures of fetal cortical neurons induces neurons to express a dopaminergic phenotype in cultures of rat or human fetal cortical neurons *(220,221)*.

2.2.2. NT-4/5

NT-4/5 is a potent survival factor for dopaminergic neurons, and of all the neurotrophins stimulates the greatest neurite outgrowth of cultured dopaminergic neurons. NT-4/5 increases cell body size, length and number of stem neurites on cultured dopamine neurons *(80,179)*. Hyman and colleagues compared the effects of BDNF, NT-3, and NT-4/5 on cultured dopamine neurons and found that of the three, NT-4/5 elicited the greatest increase in the number of surviving dopamine neurons, increased dopamine content 2.6-fold, but had no effect on dopamine uptake. NT-4/5 also protects cultured dopaminergic neurons against the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium ion (MPP^+) *(79)*. Similar to BDNF, the addition of NT-4/5 plus dopamine [or dopamine agonists] to cultures of rat fetal cortical neurons induces neurons to express a dopaminergic phenotype *(220)*.

2.2.3. NT-3

NT-3 increases the number of surviving dopamine neurons and increases dopamine uptake and content in cultures of dopamine neurons *(79)*. Similar to BDNF and NT-4/5, the addition of NT-3 plus dopamine [or dopamine agonists] to cultures of fetal cortical neurons induces a dopaminergic phenotype within a subset of cells *(220)*.

2.3. Neurotrophins: In vivo Studies

Of all the neurotrophins, BDNF has been the most extensively studied in terms of its effects on *in vivo* function and protection of dopaminergic neurons. This may simply reflect the fact that many studies have clearly identified BDNF as a component of nigrostriatal dopaminergic neurons that is present during development and persists throughout adult life, and, as mentioned above, it is one of the more potent neurotrophins for supporting and protecting dopaminergic neurons *in vitro*. While NT-4/5 may rank as a potent neurotrophic factor for dopamine neurons *in vitro*, it does not appear to have as ubiquitous a presence in the mesostriatal dopaminergic system as does BDNF. The efficacy of the neurotrophins in terms of stimulating dopaminergic function or protecting against injury *in vivo* can be ranked as follows: BDNF>NT-4/5>NT-3>NGF. The *in vivo* expression of individual neurotrophins or

neurotrophin receptors as well as the their function and protective effects are discussed below.

2.3.1. BDNF

Neurons expressing BDNF mRNA are located throughout the ventral mesencephalon in an overlapping distribution with the dopaminergic cell groups in rat and monkey *(61,160)*. Both 6-OHDA lesioning and double-labeling *in situ* hybridization studies show that a substantial subpopulation of midbrain dopaminergic neurons, themselves, express BDNF mRNA *(160,161,191)*. In turn, the vast majority of the midbrain BDNF mRNA-containing cells are dopaminergic. The greatest proportion of BDNF/dopamine cells tend to be situated in the medial substantia nigra (SN) pars compacta (SNc) and ventral tegmental area (VTA). BDNF immunoreactivity has also been demonstrated within the somata and proximal dendrites of rat midbrain neurons *(8,39,205)*. These data suggest that locally produced BDNF could influence dopaminergic neurons *via* autocrine or paracrine mechanisms.

As stated earlier, BDNF mRNA is normally undetectable in the striatum. BDNF protein, however, is found in the striatum and appears to be transported anterogradely to the striatum *via* several afferent pathways: corticostriatal, nigrostriatal, amygdalostriatal, thalamostriatal *(8,39)*. Nigral neurons also exhibit specific retrograde axonal transport of BDNF and NT-3, but not NGF, from the striatum *(9,124)*. BDNF protein levels in the striatum of young adult rats are approximately 5-7 ng/g tissue as measured by ELISA *(126,212,219)*.

As noted previously, markers for neurotrophins and neurotrophin receptors are higher in the medial than in the lateral part of the SNc in both rat *(131,160,161)* and human *(128)* brain. This may be one reason why dopaminergic neurons in the lateral SN are more susceptible to neurodegeneration (*e.g.*, Parkinson's disease), than those in the medial part of the SN.

There is evidence that BDNF injected into the striatum is taken up by dopaminergic terminals and transported retrogradely to the SN (*124)*. Therefore BDNF taken up by dopaminergic neurons just prior to a neurotoxic insult may elicit neuroprotective mechanisms. This is supported by a study performed by Levivier *et al.* in which fibroblasts genetically modified to produce BDNF were implanted into the striatum prior to an intrastriatal administration of 6-hydroxydopamine (6-OHDA), and prevented the lesion-induced loss of dopaminergic cell bodies in the SN *(103)*. The protection of dopaminergic neurons against MPP^+ or 6-OHDA neurotoxicity by BDNF was previously demonstrated in culture studies *(15,78,106,173)*. Local application of BDNF to dopaminergic cell bodies also provides protection against neurotoxic insult. For example, fibroblasts genetically modified to produce BDNF, and implanted above the intact or lesioned SN, induced sprouting of both tyrosine hydroxylase- and neurofilament- immunoreactive fibers *(109)*; or protected nigral dopaminergic neurons against MPP^+ neurotoxicity *(59)*.

Intracerebral infusion of BDNF also alters behavioral and neurochemical markers that are associated with increased dopaminergic activity. Chronic or acute infusions of BDNF into a supranigral site induce a low rate of contralateral rotational behavior following the administration of amphetamine *(3,115,167)*. Chronic supranigral infusions of BDNF elevate dopamine turnover [ratio of dihydroxyphenylacetic acid to dopamine: DOPAC/DA], dopamine release [ratio of 3-methoxytyramine to DA: 3-MT/DA] within the ipsilateral striatum *(6)*, decrease dopamine turnover in the ipsilateral SN *(115)*, and increase the spontaneous electrophysiological activity of dopaminergic neurons *(166)*. Amphetamine-induced locomotor activity is decreased after a chronic supranigral infusion of BDNF *(115)*.

Infusion of BDNF into the SN immediately after transection of the ipsilateral nigrostriatal pathway promotes survival of dopamine neurons but inhibits the expression of TH in those neurons *(67)*. Moreover, infusion of BDNF into the SN before or during chronic intrastriatal infusion of 6-OHDA does not prevent the loss of dopaminergic terminals in the striatum and does not protect against a partial loss of nigral dopamine neurons, but does reverse rotational behavior and augments dopamine metabolism *(7)*. Delivery of BDNF, intracerebroventricularly or directly into brain tissue, shows relatively poor tissue penetration and diffusion when compared to other neurotrophins (*e.g.*, NT-3 or NGF) *(9)*, due in large part to the ubiquitous presence of both catalytic and non-catalytic trkB receptors throughout the brain.

2.3.2. NT-3

Expression of NT-3 mRNA is prominent within dopaminergic cell groups of the ventral midbrain, where extensive coexistence with dopamine is observed mainly within neurons of the VTA and medial SNc *(61,160,161)*. These findings indicate that NT-3, like BDNF, may provide local trophic support to midbrain dopamine cells. Neither NT-3 mRNA nor protein have been described in the striatum. NT-3 binding, however, occurs in both the striatum and SN of adult rats *(4)*.

Supranigral infusions of NT-3 decrease dopamine turnover in the SN but not in the striatum *(115)*. NT-3 decreases body weight during chronic infusion periods, increases the frequency of amphetamine-induced contraversive rotational behavior, and attenuates amphetamine-induced locomotor activity *(115)*. Infusion of NT-3 into the SN before and during a chronic intrastriatal infusion of 6-OHDA does not prevent the loss of dopaminergic terminals in the striatum and does not protect against a partial loss of nigral dopamine neurons, but does reverse rotational behavior and augments dopamine metabolism *(7)*. Notably, infusion of NT-3 into the SN immediately after transection of the ipsilateral nigrostriatal pathway is less potent at promoting the survival of dopamine neurons than BDNF, but completely preserves TH expression *(67)*.

2.3.3. NT-4/5

Although expression of NT-4/5 mRNA has been detected in the basal ganglia by Northern Blot analysis *(183)*, its cellular distribution has not yet been elucidated. Chronic, supranigral infusion of NT-4/5 increases dopamine turnover in the striatum *(5)*. Infusion of NT-4/5 into the SN immediately after transection of the ipsilateral nigrostriatal pathway is better at promoting the survival of dopamine neurons than BDNF or NT-3, but is similar to BDNF in that it inhibits the expression of TH phenotype *(67)*. Chronic supranigral infusion of NT4/5 following transection of the medial forebrain bundle reverses the loss of calbindin-containing nigral neurons but not calretinin-containing nigral neurons *(2)*.

2.3.4. Trk B and TrkC

Both trkB and trkC mRNAs are widely distributed throughout the rat ventral mesencephalon, including all subdivisions of the SN, VTA, and retrorubral field *(6,107,119,131)*. Double-labeling studies demonstrate that essentially all mesencephalic dopamine neurons express both of the neurotrophin receptor mRNAs *(131)*. Additional non-dopaminergic perikarya in the midbrain also express the trk receptor mRNAs, and these are most likely GABAergic neurons. Immunocytochemical studies have reported labeling for the trkB receptor in cell bodies, dendrites and axons of the ventral mesencephalon *(60,204)*. Expression of trkB mRNA or immunoreactivity has also been described in human SN *(19,128)*, in similar patterns to those found in the rat. The comprehensive expression of both trkB and trkC by dopaminergic nigrostriatal neurons presents strong evidence that dopaminergic neurons have the capability to directly respond to BDNF and NT-3 *in vivo*. Given the local synthesis of the neurotrophin ligands by the dopamine cells, these neurotrophin receptor data reinforce the notion of an autocrine/paracrine mode of neurotrophin support for the dopaminergic midbrain.

Both trkB and trkC mRNAs are broadly distributed at moderate levels within numerous striatal neurons *(6,9,119,130)*. Immunoreactivity for trkB is also observed in the striatum, as well as in other regions of adult rat brain *(204)*. Two weeks following a 6-OHDA lesion of the nigrostriatal pathway, trkB mRNA expression of the catalytic form of the receptor is up-regulated in denervated striatum, when compared to expression in intact striatum (Fig. 1) *(130)*. Similarly, trkB protein levels within the striatum are increased 2-4 weeks following a transection of the ipsilateral medial forebrain bundle *(45)*. This up-regulation of trkB following dopamine denervation may indicate that dopamine negatively controls striatal trkB expression. Alternatively, it may be a compensatory response to loss of endogenous BDNF ligand, either anterogradely or retrogradely derived from the midbrain. The truncat-

ed, but not full-length, form of the trkB receptor is up-regulated during the first month following physical injury to the striatum *(202)*.

3. TGF-β/GDNF/NEURTURIN FAMILY OF NEUROTROPHIC FACTORS

Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) are structurally related, and both activate a two-component receptor complex that consists of a ligand binding GDNF family receptor (e.g., GDNFRα-1 or GDNFRα-2) and the receptor protein kinase *ret (46,83)*. GDNF mRNA is expressed in rat striatum from E20 through P7 *(154,177)* and in adult striatum of rat and human brain *(174)*. The receptor for GDNF and neurturin, GDNFRα, is localized within the SNc as well as other brain structures *(64,200, 207)*. GDNF is a glycosylated, disulfide-bonded homodimer that promotes the survival and differentiation of embryonic dopaminergic neurons, increases high-affinity dopamine uptake, and is a member of the TGF-β family *(105)*. Conditioned media obtained from fibroblasts genetically modified to express GDNF improve survival of tyrosine hydroxylase immunoreactive cells in ventral mesencephalic cultures *(85)*. When infused into *in oculo* transplants of fetal ventral mesencephalic tissue, GDNF stimulates growth of transplanted dopaminergic neurons *(177)*. GDNF rescues surviving dopaminergic neurons following 6-OHDA or axotomy-induced lesions. However, protection is not permanent if GDNF treatment is terminated *(108)*. Infusion of GDNF into the SN up-regulates dopaminergic function in both young and aged animals. For example, intranigral injections of GDNF increase locomotor activity, basal dopamine levels in the striatum, and both K^+- and amphetamine-induced overflow of dopamine in the striatum of aged rats *(22,70,98)*. In young adult rats, intranigral injections of GDNF induce sprouting of nigral dopaminergic neurons and elicit an increase in nigral and striatal dopamine turnover that persists for at least 3 weeks following the injection *(77)*. Injections of GDNF into the SN not only alter dopamine neurochemistry but also protect dopaminergic neurons against neurotoxic and traumatic injury to the nigrostriatal system. For example, intranigral injections of GDNF protect dopaminergic neurons against 6-OHDA toxicity *(87,88,181)*, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity *(62,184)*, methamphetamine toxicity *(31)*, and axotomy-induced neurodegeneration of dopaminergic neurons *(18)*. Continuous release of low levels of GDNF into a perinigral region protects nigral dopaminergic neurons against axotomy-induced lesion and improves pharmacological rotational behavior by non-dopaminergic mechanisms *(188)*. Intermittent injections of GDNF into a perinigral region prevents 6-OHDA induced degeneration of nigral dopamine neurons *(152)*. Delivery of GDNF to perinigral regions using viral vectors

has been shown to halt 6-OHDA induced degeneration of dopamine neurons *(34,111)*.

The more recently discovered neurturin (NTN) has been shown to promote the survival of dopaminergic neurons and protect dopaminergic neurons against 6-OHDA neurotoxicity *(75)* and axotomy of the medial forebrain bundle (189). The NTN receptor, GDNFRα, is expressed in and around nigral dopaminergic neurons *(75)*. Although GDNF and NTN bind to the same receptor complex, NTN shows a lower efficacy than GDNF for protecting dopaminergic neurons against 6-OHDA neurotoxicity after infusing either one intrastriatally or intracerebroventricularly, and this may be related to NTN's poor solubility and diffusion properties at a physiologic pH *(147)*.

Transforming growth factor-β (TGF-β) is structurally related to GDNF and NTN. Both TGF-β2 and TGF-β3 are expressed in the vicinity of dopaminergic neurons during embryonic development, and the addition of these two factors to cultured dopaminergic neurons prevents neuronal death *(144)*. The TGF-β protein family activates a class of serine/threonine kinase receptors *(157)* and these receptors are found in the caudate nucleus, putamen, ventral pallidum, and nucleus accumbens during development and in adult rat brain *(190)*.

4. EPIDERMAL GROWTH FACTOR (EGF)/ TRANSFORMING GROWTH FACTOR (TGF-α)

EGF and TGF-α are two members of a structurally related family of polypeptide growth factors that also includes heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin and epiregulin *(100,142)*. All of these molecules exert their biological effects *via* interaction with the EGF receptor, a transmembrane protein tyrosine kinase *(142)*. Several lines of evidence suggest that both EGF and TGF-α can provide trophic support for mesencephalic dopaminergic neurons.

EGF promotes the survival of cultured fetal dopaminergic neurons *(29,55,89)* and protects cultured dopaminergic neurons against glutamate toxicity *(30)*. EGF also protects cultured midbrain dopaminergic neurons against MPP^+ neurotoxicity and promotes the recovery of MPP^+-damaged neurons *(138,156)*. Since glial cell proliferation is required for some of these effects, it is thought that the actions of EGF on midbrain dopamine cells may be mediated indirectly *via* glia. *In vivo*, EGF mRNA and EGF-like immunoreactivity are expressed in rodent basal ganglia *(50,99)*, although at very low levels. EGF receptor mRNA is also present within the basal ganglia, including neurons of the SNc, VTA, and striatum of rat *(96,97,162)*. Interestingly, we have shown that the EGF receptor is expressed by subpopulations of dopaminergic neurons in the medial nigra and VTA *(97,162)* and by GABAergic interneurons in the striatum *(96)*. Thus, in contrast to findings *in vitro*, it appears that dopaminergic (and GABAergic) neurons *in vivo* could respond directly to EGF receptor ligands. In lesion

studies *in vivo*, intracerebroventricular infusion of EGF increases the survival of dopamine neurons following a unilateral transection of the nigrostriatal pathway *(140,192)* or in MPTP-treated mice *(65)*, suggesting a neuroprotective or neurorestorative role for the growth factor in animal models of Parkinson's disease.

Studies of TGF-α in the mesostriatal system have led to the speculation that this EGF receptor ligand may provide local or target-derived trophic support to midbrain dopamine neurons. Similar to EGF, TGF-α increases the number and neurite outgrowth of cultured embryonic dopaminergic neurons, perhaps indirectly *via* glia *(1)*. In contrast to EGF, TGF-α mRNA is expressed at particularly high levels in the rodent striatum, including both the caudate-putamen and nucleus accumbens *(95,99,158,159,201)*, where the cellular localization of its mRNA or protein has been described in both neurons and astrocytes *(51,159,199,201)*. Striatal TGF-α is developmentally regulated, with higher levels of expression present perinatally *vs.* adulthood *(95,97,159)*. These high levels of TGF-α expression, coupled with the low levels of EGF, suggest that TGF-α is the major endogenous ligand for the EGF receptor in the mesostriatal system. Although TGF-α mRNA is not present in the adult ventral mesencephalon, it is expressed in midbrain regions prenatally *(97)*. Given the presence of the EGF receptor both prenatally and postnatally in a subset of the dopaminergic neurons *(97,162)*, as well as the above noted trophic effects of TGF-α on dopamine cells *in vitro*, an autocrine or paracrine role for TGF-α in the survival or differentiation of developing dopamine neurons is a distinct possibility. Retrogradely derived TGF-α from the striatum would be another likely source of trophic support for midbrain dopaminergic neurons. In any event, the importance of TGF-α in the survival and/or differentiation of dopamine cells is underscored by the recent finding that approximately 50% of nigral dopamine neurons are absent in TGF-α-deficient mice *(21)*.

Our recent work on the regulation of TGF-α and EGF receptor expression in the nigrostriatal system reveals that TGF-α mRNA is decreased in the striatum ipsilateral to a 6-OHDA lesion of the nigrostriatal pathway (Fig. 4). Expression of striatal EGF receptor mRNA, however, remained unchanged. These data indicate that striatal TGF-α is not upregulated as a compensatory response to nigral dopamine neuron injury and that dopaminergic afferents may normally promote striatal TGF-α expression. Consonant with the above findings, no increase of EGF binding is detectable in the striatum of monkeys following MPTP treatment *(194)*. However, in brains from Parkinson's disease patients, EGF binding is elevated at anterior levels of the dorsal striatum *(194)*, but remains unaffected in the mesencephalon *(193)*. The latter finding raises the possibility that human midbrain EGF receptor-expressing cells, which may characterize a subset of dopaminergic neurons in rat, may be spared in Parkinson's disease.

Finally, it should be noted that two other EGF receptor ligands have recently been detected in rodent basal ganglia. Amphiregulin mRNA is found within a small subpopulation of striatal cells that exhibits a patchy hybridization pattern *(163)*. Expression of HB-EGF mRNA, although not normally present in the striatum, is slightly induced in the dorsal caudate-putamen following excitotoxic seizures *(134)*. The possible functional roles of these growth factors in the dopaminergic mesostriatal system remain to be determined.

5. FIBROBLAST GROWTH FACTOR (FGF)

Acidic FGF (aFGF or FGF-1) and basic FGF (bFGF or FGF-2) are members of a family of nine known fibroblast growth factors [FGF-1 through FGF-9] *(47)*. Four FGF receptors (FGFR) have been identified and both aFGF and bFGF bind with high affinity to FGFR-1. FGFR-1 is expressed primarily by neurons *(196)* while FGFR-2 and FGFR-3 are expressed mainly by glia *(11)*. FGFR mRNA is moderately expressed in the SN and VTA in rat brain *(196)*. Basic FGF is localized to the SN of rat, monkey, and human brain *(13,35,185)*. While bFGF is expressed in the rat ventral mesencephalon from E16 onward, aFGF is not observable until P20 and later *(14)*. Injection of ^{125}I-bFGF into the striatum labels the ipsilateral SN, suggesting that bFGF binds to FGF receptors in the striatum and is retrogradely transported to the SN *(53)*. Both bFGF and aFGF mRNAs are increased in the striatum of mice following treatment with MPTP *(102)*. Postmortem analysis of bFGF immunoreactivity in the SN of normal aged brain and brains from Parkinson's patients are significantly different. In the normal aging human brain, 82% of nigral dopaminergic neurons are bFGF-immunoreactive while only 12% of the remaining nigral dopaminergic neurons are bFGF-immunoreactive in brains of patients diagnosed with Parkinson's disease *(186,187)*. In contrast, postmortem analysis of the bFGF receptor, FGFR-1, in normal and Parkinsonian brain show that most residual dopaminergic neurons also express FGFR-1 immunoreactivity *(195)*. Thus, in Parkinson's disease dopaminergic neurons may lose the neurotrophic support of bFGF, however, the surviving neurons maintain expression of its receptor.

The identification and localization of FGF and FGF receptors within the basal ganglia prompted researchers to test whether or not they are important factors for growth and survival of dopaminergic neurons. It was initially determined that bFGF promoted the survival and growth of cultured dopaminergic neurons *(52,54, 89,117)*. The neurotrophic mechanism of the fibroblast growth factors remains unknown, however, several studies suggest that their neurotrophic action is mediated by glia *(48,138)*. Subsequent studies showed that bFGF enhanced dopaminergic neuron survival indirectly through glial cells *(49,89,117,214)* and that the glial-mediated effect of bFGF on dopaminergic neurons does not involve the secretion of GDNF from glia *(76)*. Similar to other neurotrophic factors, bFGF not only possess-

es neurotrophic support for dopaminergic neurons *in vitro*, but also neuroprotects dopaminergic neurons *in vitro* and *in vivo*. For example, bFGF protects dopaminergic neurons against glutamate toxicity *(30)* and against MPP^+ neurotoxicity *in vitro (138)* and *in vivo (32,43)*; glial cell proliferation was required for *in vitro* protection against MPP^+ *(138)*. Basic FGF enhances survival and sprouting of fetal dopaminergic neurons implanted into the denervated striatum *(118,175,182)*. Acidic and basic FGF protect dopaminergic neurons against MPTP toxicity in young but not aged mice *(42,136)*. In addition to aFGF and bFGF, another member of the FGF gene family, FGF-8, has been found to play a key role in intercellular signaling at various developmental time points. FGF-8 is required in combination with sonic hedgehog (see below) for the induction of dopaminergic neurons in the ventral midbrain during embryonic development *(206)*.

6. SONIC HEDGEHOG (Shh)

Sonic hedgehog is synthesized as a 45 kDa precursor protein that undergoes proteolytic cleavage to yield a 20 kDa amino-terminal cleavage product (Shh-N), which remains mostly cell-associated, and a carboxy-terminal cleavage product (Shh-C), which diffuses more freely. Only the amino-terminal cleavage product (Shh-N) has been implicated in mediating signaling activity *(143)*. Because Shh-N is cell-associated, direct cell-cell contact is thought to be an important component in mediating Shh signaling activity. Ye *et al.* provided evidence that Shh and FGF-8 are required for the induction of midbrain dopaminergic neurons during normal development, and that the combination of Shh and FGF-8, but neither one alone, can induce neurons with a dopaminergic phenotype in ectopic regions along the anterior neural tube *(206)*. Shh is capable of inducing dopaminergic neuron phenotype in chick mesencephalic explants *(197)*, rat explants *(81)*, and promotes the survival as well as protects cultured dopaminergic neurons against MPP^+ neurotoxicity *(121)*. Biochemical evidence suggests that patched (*ptc*) is the receptor for Shh *(114,176)* and it would be interesting to determine whether or not the *ptc* receptor is localized to dopaminergic neurons in the ventral midbrain.

7. PLATELET-DERIVED GROWTH FACTOR (PDGF)

Platelet-derived growth factor (PDGF) promotes survival of cultured dopaminergic neurons. The isoform PDGF-BB, and not isoform PDGF-AA, provides trophic support for cultured human ventral mesencephalic tissue *(127)*, and protects cultured dopaminergic neurons against 6-OHDA toxicity *(141)*. In cultures of rat and human dopaminergic neurons, PDGF beta-receptors are found on approximately 70% of tyrosine hydroxylase positive neurons *(135)*.

8. NEUREGULINS AND NEUREGULIN RECEPTORS

Neuregulins comprise a large group of structurally homologous polypeptide growth factors that are related to members of the EGF family *(23,26,101,112,113, 217)*. Described independently as acetylcholine receptor inducing activity (ARIA), glial growth factors (GGF), heregulins, or neu differentiation factor (NDF), it is now known that all of these factors and other isoforms arise from alternative splicing of a single gene. The EGF-like domain shared by the members is thought to be responsible for receptor binding and subsequent biological activities. Neuregulins interact directly or indirectly with the erbB family of homologous, transmembrane protein tyrosine kinase receptors (erbB2, erbB3 and erbB4), which also includes the EGF receptor (also known as erbB1, although neuregulins cannot directly bind the EGF receptor). Upon ligand binding, erbB receptors can form homodimers or heterodimers with each other, leading to activation and subsequent intracellular signaling *(23,25)*. However, neuregulins can directly bind to only the erbB3 (kinase-defective) and erbB4 receptors, which can then heterodimerize with erbB2 and erbB1 to activate and/or potentiate subsequent signaling activity. Recently, additional neuregulin-like genes have been cloned, now termed neuregulin-2 *(24,27,33,71)* and neuregulin-3 *(216)*, that can bind and activate the erbB3 and erbB4 receptors.

In addition to trophic effects of neuregulins reported in various non-neuronal cell types and organs (particularly in heart development), the most well-known functions associated with the nervous system include roles in glial cell proliferation, differentiation and survival, neuromuscular acetylcholine receptor induction, and neuronal migration and survival. The functions of neuregulins in the mature brain, however, and particularly in the basal ganglia, are unresolved. With respect to the dopaminergic mesotelencephalic system, "weak" neuregulin expression was reported in the postnatal caudate-putamen *(40)*, and neuregulin-3 mRNA has been detected in the caudate-putamen and SN by Northern blot analysis *(216)*.

To begin to investigate the role of neuregulins and their erbB receptor family in the dopaminergic mesostriatal system, we have recently examined the expression of erbB4 mRNA within the normal and lesioned ventral midbrain. We find that erbB4 mRNA is expressed within a substantial population of cells within the SN and VTA of both rodent (Fig. 2) and monkey, exhibiting a striking spatial overlap with the mesencephalic dopaminergic cell groups *(164)*.

Expression of erbB3 mRNA, in contrast, is primarily restricted to presumed glia mainly in the cerebral peduncles in the ventral mesencephalon *(164)*. Both neurotoxin lesioning and double-labeling *in situ* hybridization studies demonstrate a high degree of erbB4/dopamine coexistence in ventral mesencephalic neurons of both rat and monkey *(164,165)*. These results raise the possibility that the dopaminergic neurons could be responsive to the trophic

effects of neuregulins. Indeed, our preliminary findings using *in vivo* microdialysis indicate that a single supranigral injection (10 μg) of the neuregulin heregulin-β1 induces a substantial increase in dopamine overflow in the ipsilateral striatum *(164)*. This is among the first *in vivo* demonstrations of neuregulin action in the brain and provides the first evidence that neuregulins can affect dopaminergic nigrostriatal function. We also find that neuregulin treatment protects the human neuroblastoma dopaminergic cell line SK-N-MC from both oxidative and metabolic insults *(165)*. Taken together, these findings raise fundamental questions as to the role of neuregulins in development and maintenance of the dopaminergic mesotelencephalic system, and suggest that neuregulins may have trophic capabilities for dopaminergic neurons. Functional studies of neuregulin actions in normal nigrostriatal system as well as in animal models of Parkinson's disease are currently in progress in our laboratories.

9. OTHER DOPAMINERGIC TROPHIC FACTORS

Engele *et al.* demonstrated that conditioned media from mesencephalic glial cells possesses factors that promote the survival and differentiation of cultured dopaminergic neurons *(49)*. Dong *et al.* *(44)* showed that human fetal mesencephalic dopaminergic cells survived better when co-cultured with striatal glial cells [target-derived glia] than with mesencephalic glial cells [non-target derived]. This suggests that target-derived glia may provide better trophic support for dopaminergic neurons than non-target glia. Thus, factors that induce a proliferation of glial cells may indirectly affect the survival and differentiation of dopaminergic neurons by increasing the pool of glial-derived neurotrophic factors. Both EGF and the FGFs are examples of glial mitogens that enhance the survival of cultured or transplanted dopaminergic neurons through neurotrophic mechanisms mediated by glial cells. Interestingly, interleukin-1β has been shown to induce axonal sprouting of dopaminergic neurons in lesioned animals, however, this event may be mediated by an induction of bFGF within astrocytes *(72)*. Insulin-like growth factor-1 (IGF-1) promotes the survival of cultured dopaminergic neurons *(16,17,89,91)* and IGF receptors have been localized within the basal ganglia *(56)*. Ciliary neurotrophic factor (CNTF) has been shown to prevent the loss of nigral dopaminergic neurons following a lesion of the nigrostriatal pathway *(66)* and the CNTF-α receptor has been localized to neurons in the SN of monkey brain *(94)* and rat brain *(107)*. Nicotine induces the expression of BDNF and bFGF, as determined by RNase protection assay, in rat striatum, and nicotine treatment in mice or rats prevents MPP+ or methamphetamine-induced neurotoxicity, respectively *(110)*. Thus, it appears that in addition to application of exogenous neurotrophic factors themselves, agents that induce or up-regulate endogenous neurotrophic molecules in the nigrostriatal system may provide an alternative means by which dopaminergic neurons could access trophic support.

10. NEUROTROPHIN KNOCKOUTS AND NEUROTROPHIN RECEPTOR KNOCKOUTS

The use of animals with targeted gene disruption for neurotrophins, neurotrophin receptors, and other neurotrophic factors has demonstrated that survival and differentiation of some peripheral and central neurons are highly dependent upon the expression of neurotrophic factors during development. How does gene disruption [knockouts] of specific neurotrophins or neurotrophin receptors affect the development of central dopaminergic neurons? It is important to first consider that two neurotrophin receptors, trkB and trkC, are known to be co-localized in dopaminergic neurons in the ventral mesencephalon *(6,119,131)*. This means that at least one neurotrophin that binds with high affinity to trkB or trkC can remain active following a knockout of single gene encoding any one of the neurotrophins or neurotrophin receptors. Several studies have demonstrated that disruption of a gene encoding for any one of the neurotrophins or trk receptors did not have deleterious effects on the development of central dopaminergic neurons *(82,84)*. One argument for the lack of an effect on dopaminergic neurons following a single gene knockout for one neurotrophin or neurotrophin receptor is that the remaining active neurotrophin[s] may provide compensatory neurotrophic support. However, mice with double knockouts of genes encoding the catalytic domains of both trkB and trkC also show normal development of central dopaminergic neurons although these animals typically do not live beyond the fifth postnatal day *(168)*. The double trk receptor knockout provides a model in which the neurotrophins are unable to affect dopaminergic neurons *via* conventional receptor binding mechanisms. With regard to the double trk receptor knockout study, it should be pointed out that deficits produced by mutations of only the catalytic domain of the trk receptors may not be as severe as those produced by a mutation that deletes all the proteins encoded by the trk gene *(104)*. At this time, however, there is no known signaling function for truncated trkB or trkC receptors on dopaminergic neurons. Mice with a null mutation of the p75 receptor also show normal development of the SN and striatum *(139)*. The results of the single and double knockout studies strongly imply that neurotrophins may not be essential for the survival, differentiation, and growth of central dopaminergic neurons during embryonic and early postnatal development. Indeed, we already mentioned that developing dopaminergic neurons are responsive to a multitude of other endogenous neurotrophic factors that are important for the differentiation, survival, and maturation of dopaminergic neurons.

While mutations of genes encoding neurotrophins or neurotrophin receptors do not have an apparent effect on the development of central dopaminergic neurons, the development of central dopaminergic neurons *in vivo* can be adversely affected in mice deficient for other neurotrophic factors. For example, mice with a mutation that reduces, but does not eliminate,

FGF-8 levels generate a population of midbrain dopaminergic neurons that are severely reduced in numbers when compared to normal littermates *(120,206)*. Another study, as described previously, using mice with a null mutation for the gene encoding TGF-α, a member of the EGF family that activates the EGF receptor, demonstrated a 50% reduction of dopaminergic neurons in the SN *(21)*. The fact that 50% of dopaminergic neurons survived after this mutation may be attributed to experimental evidence that not all dopaminergic neurons express the EGF receptor *(97,162)*, and, thus, presumably are not responsive to TGF-α. These findings may also illustrate the versatility of dopaminergic neurons in terms of their dependency on heterologous lines of neurotrophic support during development.

11. AGING AND NEUROTROPHIC FACTOR EXPRESSON IN THE NIGROSTRIATAL SYSTEM

Much of the evidence that supports a decline of neurotrophic activity in the aging nigrostriatal system is derived from studies that have examined the *in vitro* development of embryonic dopaminergic neurons co-cultured with striatal extracts obtained from young and old brain, or from neural transplantation studies. For example, Carvey and co-workers noted that the compensatory increase in striatal trophic activity following a 6-OHDA lesion is lower in aged than in young rats *(28)*. Similarly, Kaseloo and colleagues *(86)* reported that striatal extracts taken from the injured striatum of aged rats possessed a diminished capacity for inducing neurite outgrowth in cultures containing the SH-SY5Y cell line [dopamine-producing neuroblastoma cell line]. In aged rats with long-term 6-OHDA lesions, transplants show poor survival and function unless neurotrophic supplements (*e.g.*, Schwann cells) are co-grafted with transplants *(36-38)*. In a recent study we observed that striatal BDNF protein levels increase following a 6-OHDA lesion of the nigrostriatal pathway in both young and aged rats, however, the increase is significantly greater in denervated striatum of young rats *(212)*. We also have preliminary data showing that NT-3 mRNA expression is reduced in the ventral midbrain of aged rats (Fig. 6), suggesting that production of neurotrophins by mesencephalic dopamine neurons themselves also diminishes with age.

The results of these studies indicate that compensatory neurotrophic mechanisms that occur following a degenerative lesion may decline with age and, likewise, that the denervated striatum of an aged animal may provide a poorer trophic environment for transplants than the denervated striatum of a young animal. With regard to neural transplantation studies, this is a significant statement because most of the basic scientific research has been performed in young adult animals with experimental Parkinson's disease while human transplant recipients are typically aged patients with advanced Parkinson's disease. Freed *et al.* recently reported that younger Parkinson's patients respond more favorably to human embryonic dopamine

cell transplants than older patients in terms of their post-operative PET scan results and neurological assessments *(57)*. The age disparity between animal and human transplant recipients may prove to be a crucial variable in terms of explaining why animal studies typically yield more promising experimental results than clinical transplant studies. Similarly, in order to develop effective strategies for preventing age-related neurodegenerative disorders, it will be important to determine whether or not there is a general decline of neurotrophic factor activity or responsiveness to neurotrophic factors in the aging brain.

12. NEURAL TRANSPLANTS AND NEUROTROPHIC FACTORS

Transplantation of embryonic dopaminergic neurons into adult brain has been a valuable neurobiological tool for studying basal ganglia function and the development of dopaminergic neurons, as well as a potential therapeutic source of dopaminergic neuron replacement in neurodegenerative disorders such as Parkinson's disease *(38,208)*. As a therapy for Parkinson's disease, several problems have been encountered with transplants of human embryonic dopaminergic tissue into the Parkinsonian brain, and those problems are related to the survival of transplanted neurons and the establishment of functional contacts between the transplanted neurons and the host brain. For example, transplants of embryonic dopaminergic neurons alone into the brains of Parkinson's patients typically show a low-to-moderate survival rate, sparse functional contacts with the host brain, and limited therapeutic value. Research in this field has been redirected toward improving the survival of transplanted neurons by supplementing embryonic transplants with neurotrophic substrates that have been shown to increase the *in vitro* survival of dopaminergic neurons. The following discussion describes the neurotrophic environment of embryonic dopaminergic cells implanted into the striatum of animals with experimental parkinsonism, and provides examples of studies that have used combinations of neural transplants and neurotrophic factors as a strategy to improve the survival of embryonic neurons transplanted into adult brain.

During the first month after embryonic dopaminergic neurons are implanted into adult nervous tissue, neurotrophic markers change dynamically within the transplant and also within the host tissue. During normal development the expression of BDNF mRNA in dopaminergic neurons typically decreases during late embryonic and early postnatal periods. However, BDNF mRNA expression in *in oculo* transplants of embryonic dopaminergic neurons does not decrease but is maintained at constant levels for 6 weeks after transplantation *(178)*. We also observe a constant expression of BDNF mRNA in intrastriatal transplants of embryonic dopaminergic neurons throughout the first month of transplant development as seen in figure 6 *(213)*. Prominent expression of trkB mRNA is present immediately after transplantation and shows a progressive reduction during the first month (Fig. 7) *(213)* or month-and-

a-half *(178)*. On the other hand, immunostaining for the trkB receptor is reportedly not present in fetal ventral mesencephalic transplants until two weeks after transplantation *(153)*. The results of these studies show a discordance between protein and mRNA expression: trkB mRNA expression is evident weeks before trkB immunoreactivity is present in transplants. It is interesting that trkB immunostaining is not detectable in transplants of embryonic dopaminergic neurons until two weeks after transplantation because fiber outgrowth from transplants can be optimally stimulated by exogenous BDNF by delaying its infusion until two weeks after transplantation *(210)*. The results of these studies provide evidence that immature dopaminergic neurons may be more responsive to BDNF at a developmental time point when trkB receptors are abundantly expressed on dopaminergic neurons.

Not only is there a dynamic change in markers for neurotrophic activity within the transplant, but markers of neurotrophic activity within the host tissue surrounding the transplant also appear to be influenced by the presence of a transplant. As stated earlier, following a lesion of the nigrostriatal pathway markers for the trkB receptor in the denervated striatum are up-regulated when compared to trkB markers in the intact striatum *(45,130)*. Striatal trkB mRNA remains elevated immediately after embryonic dopaminergic neurons are implanted into the denervated striatum, however, by the 4th week trkB mRNA is normalized in denervated/transplanted striatum while animals without transplants maintain an up-regulation of trkB mRNA expression in the denervated striatum *(213)*.

One means of improving the survival and functional integration of transplanted dopaminergic neurons is to administer a neurotrophic supplement with the transplant that is known to promote the differentiation, survival, and growth of dopaminergic neurons. The following discussion lists the results of studies that have added neurotrophic factors to transplanted dopaminergic neurons.

Neurotrophic factors have been added as supplements to transplants of embryonic dopaminergic neurons using several different techniques: 1) pre-incubation of embryonic cells or tissue with the neurotrophic factor, 2) single or intermittent bolus injections of neurotrophic factor into, or adjacent to, the transplant *via* syringe needle, 3) chronic intracerebral delivery using osmotic pumps, or 4) co-grafting embryonic dopaminergic neurons with genetically modified cells that express and release neurotrophic factors. The neurotrophin NT-4/5 injected intermittently into a site adjacent to the transplant promotes fiber outgrowth and enhances functional efficacy of dissociated cell suspension transplants obtained from the fetal ventral mesencephalon *(68)*. Intermittent injections of BDNF directly into the transplant enhances function rather than survival of fetal ventral mesencephalic transplants *(141)*, while continuous infusion for 4 weeks enhances both function and fiber outgrowth *(209)*. GDNF enhances the survival and fiber outgrowth from fetal ventral mesencephalic trans-

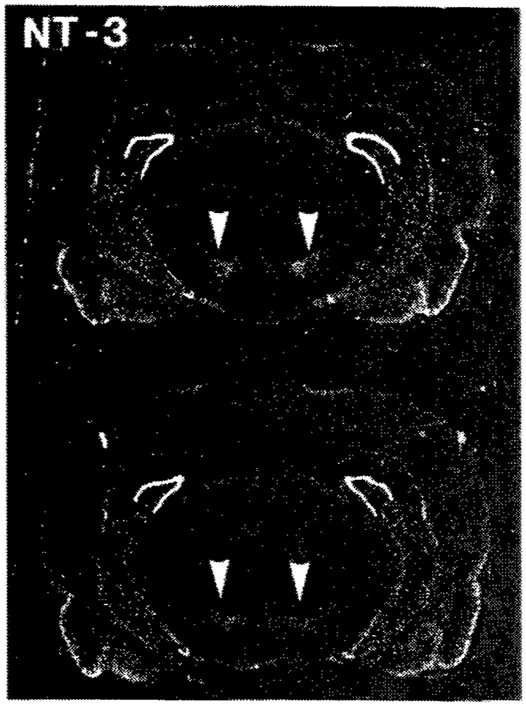

Fig. 6. NT-3 mRNA expression in the ventral midbrain of young and old rat brain. Film autoradiogram shows the *in situ* hybridization localization of NT-3 mRNA in the normal midbrain of young (4 month old, **top**) and old (24 month old, **bottom**) rats. White arrowheads point to the substantia nigra /ventral tegmental area; note lower density of NT-3 hybridization in this region of the old rat brain.

plants after pre-incubating the transplant with GDNF or infusing GDNF into the transplant site intermittently or chronically *(10,146,169,211)*. Figure 5 shows an example of how an infusion of GDNF into an intrastriatal transplant of embryonic ventral mesencephalic tissue dramatically improves the number of surviving transplanted dopaminergic neurons.

Another member of the GDNF family, neurturin (NTN), enhanced the survival of dopaminergic neurons in intrastriatal transplants of embryonic ventral mesencephalon after intermittent infusion of NTN into the transplant for a three week period *(148)*. Wang and colleagues used a combination of intranigrally placed fetal ventral mesencephalic grafts and GDNF to reconstruct dopaminergic nigrostriatal projections in rat brain *(198)*. Acidic FGF increased fiber outgrowth without increasing the number of surviving transplanted embryonic dopaminergic neurons, while bFGF increased both fiber outgrowth and the number of transplanted embryonic dopaminergic neurons *(63)*. Basic FGF enhances survival and sprouting of embryonic dopaminergic neurons implanted into the denervated striatum using direct

injections of bFGF or genetically modified fibroblasts that express bFGF *(118,175,182)*. Similar to the effects of bFGF on cultured dopaminergic neurons reported earlier, the increased survival of transplanted embryonic dopaminergic neurons treated with bFGF is associated with a concomitant increase in glial proliferation within the transplant *(215)*.

Earlier in this chapter we provided examples of how neurotrophic factors are differentially expressed during normal development with some neurotrophic factors highly expressed during embryonic and early postnatal development; and other neurotrophic factors showing a delayed postnatal expression. The time course of neurotrophic factor expression may be an important component for properly sculpting the growth of immature neurons so that they may survive and grow to form functional contacts with their targets. For transplanted neurons, this important sequence of neurotrophic activity is most likely lacking and therefore the transplanted neurons do not receive the proper signals for successful growth and integration into the neural circuitry of the host brain. We recently performed a study where the neurotrophin BDNF was infused into transplants of embryonic dopaminergic neurons, but purposely delayed the infusion until a developmental time point when BDNF is normally expressed at high levels in the striatum, the natural target for developing nigral dopaminergic neurons. While the delayed infusion did not enhance the survival of transplanted dopaminergic neurons, it did improve the fiber outgrowth of the transplanted neurons *(210)*. The results of this study suggest that BDNF may act as a target-derived neurotrophic factor for developing dopaminergic neurons, and that a postnatal development window may exist when dopaminergic neurons are responsive to the effects of BDNF in terms of stimulating dopaminergic fiber growth. Effective strategies for improving transplant survival and host reinnervation will most likely utilize several neurotrophic factors in combination with one another so that survival, growth, and reinnervation are optimized.

13. STRATEGIES FOR DOPAMINE NEURON PROTECTION OR RESCUE

Many of the neurotrophic factors discussed above have been shown to be very potent survival and growth factors when administered directly to cultured dopaminergic neurons. However, applying neurotrophic factors to neurons within the CNS of living organisms poses a different set of problems. To date, most *in vivo* studies have reported beneficial effects of neurotrophic factors after using invasive neurosurgical techniques as a means to apply neurotrophic factors directly to CNS target sites. These invasive techniques are used because most neurotrophic factors are large proteins incapable of crossing the blood-brain barrier, and this limitation renders most common routes of drug administration (*e.g.*, oral, subcutaneous, or intravenous) as ineffective for delivery to damaged or degenerating CNS sites. Techniques for

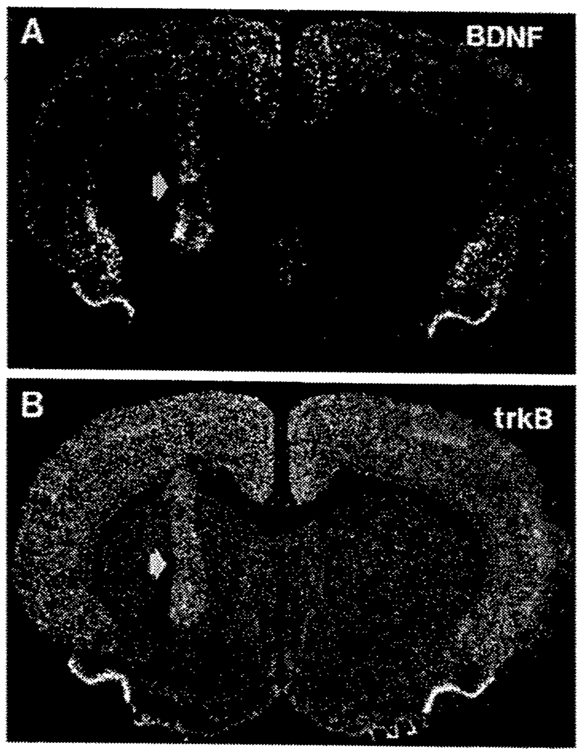

Fig. 7. Autoradiograms of adjacent sections showing robust expression of BDNF (A) and trkB (B) mRNAs in a fetal mesencephalic graft (arrows) 2 weeks after implantation into dopamine-denervated rat striatum. Similar levels of BDNF expression are maintained in the grafts during the entire first month post-transplantation, whereas trkB mRNA levels in the grafts decline over time.

direct intracerebral delivery of neurotrophic factors include syringe needles for acute injections or injector cannulae attached to mechanical pumps, osmotic pumps, or refillable subcutaneous pumps for chronic delivery; mechanical pumps and refillable subcutaneous pumps have been used to test the clinical efficacy of NGF *(69,92,133)*. Intracerebroventricular administration of most neurotrophic factors is problematic because penetration into brain tissue is typically poor and diffusion from the ventricles is limited to periventricular regions *(9)*. Another technique that is currently being tested as a means to introduce neurotrophic agents into the CNS is the insertion of the gene[s] coding for neurotrophic factors into targeted brain regions. This can be achieved by several methods. First, genetically modified cells [stem cells, immortalized cell lines, or fibroblasts] that express neurotrophic factors can be implanted into target CNS sites, and these modified cells can then synthesize and release specific neu-

rotrophic factors or neurotransmitters directly into the surrounding brain tissue. Second, viral vectors can be used to infect nervous tissue and insert into the genome of the infected tissue a genetic code that promotes the expression of a neurotrophic factor *(74,125,145)*. The latter technique was used in earlier mentioned studies that used viral vectors to insert a genetic code into infected midbrain cells that promoted the synthesis of GDNF, which ultimately protected dopaminergic neurons against a subsequent neurotoxic insult *(34,111)*.

One noninvasive technique that is being developed in order to circumvent the problem of crossing the blood-brain barrier is to attach a neurotrophic factor to a molecule that is actively transported across the blood-brain barrier. Transferrin receptors are located on brain capillary endothelium and are known to transport receptor-bound particles across the blood-brain barrier by the process of transcytosis. Antibodies directed against the transferrin receptor are transcytosed across the blood-brain barrier and accumulate in brain tissue *(137)*. Therefore these antibodies may be used as vectors to transport conjugated proteins across the blood-brain barrier. For example, NGF conjugated to OX-26, an antibody directed against the transferrin receptor, is capable of crossing the blood-brain barrier in a dose-dependent fashion and protects striatal cholinergic neurons in an animal model of Huntington's disease *(93)*. Similarly, pegylated BDNF conjugated to OX-26 and administered intravenously to rats crosses the blood-brain barrier and protects hippocampal neurons after ischemia *(203)*. In a similar manner dopaminergic neurotrophic factors could be conjugated to antibodies directed against the transferrin receptor, or conjugated to ligands with high affinity for the transferrin receptor, and transported across the blood-brain barrier to affect forebrain or midbrain target sites as a means to prevent or halt neurodegeneration associated with Parkinson's disease.

As discussed above, dopaminergic neurons do not appear to be dependent upon any one neurotrophic factor for survival or normal development. However, what remains to be determined is whether or not the chronic loss of any one neurotrophic factor affects the long-term survival and function of dopaminergic neurons throughout the life of the organism. *In vivo* neurotrophic support for dopaminergic neurons may require the activity of several different neurotrophic factors operating independently or in conjunction with one another in order to sustain normal function and activity of the neuron. Therefore, therapies to restore function in degenerating dopaminergic neurons may require multiple neurotrophic factors or factors that stimulate the expression of more than one neurotrophic factor.

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The Long-term Survival of Cultured Dopaminergic Neurons is Promoted by the Neuregulin GGF2

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INTRODUCTION

We previously reported that the growth/differentiation factor glial growth factor-2 (GGF2), an isoform derived from the neuregulin-1 gene, protected cultured dopaminergic neurons against serum deprivation and 6-hydroxydopamine-induced degeneration (Zhang et al., '01). However, the normal short-term survival of the developing dopamine cells was not affected by GGF2 treatment. The current study was conducted to determine if GGF2 could promote long-term survival of dopaminergic neurons *in vitro*, and to assess the possible role played by mesencephalic glia. Cells derived from the fetal midbrain were plated at low density and were given 5% fetal bovine serum for 48 hours, after which cells were changed to fresh, serum-free culture medium and treated with either GGF2 (100ng/ml) or vehicle. Numbers of tyrosine hydroxylase (TH)-immunoreactive (dopaminergic) neurons and glial fibrillary acidic protein (GFAP) - immunoreactive (glial) cells were subsequently assessed at 7, 14, and 21 days *in vitro* (DIV). Western blots were performed in parallel cultures to detect ErbB receptor and GFAP protein expression in both control and GGF2-treated cultures. Also, we used *in situ* hybridization to determine if expression of ErbB4 and TH mRNAs is co-distributed in the E14 mesencephalic flexure. Our results indicate that GGF2 promotes the long-term survival of dopaminergic neurons and regulates expression of its' receptors in developing midbrain cultures, possibly via glial mechanisms.

Methods

Midbrain cultures:
Ventral mesencephalons were dissected using sterile techniques from embryonic day (E) 14 Sprague Dawley rat embryos (taken from timed-pregnant dams) and pooled in an oxygenated, cold, sterile calcium-magnesium-free (CMF) buffer. Cell suspensions of embryonic mesencephalic tissue were prepared through a series of CMF rinses, incubated in 0.125% trypsin, rinsed in CMF again, and triturated in 0.004% DNase to disperse the cells into solution. Cells were plated on poly-D-Lysine-coated culture plates at a concentration of 2500cells/µl or 5000 cells/ml using the microisland method (Takeshima et al., '96; Sortwell et al., '00). All cell cultures were kept in a water-jacketed tissue culture incubator at 37oC with 5% CO2/95% air.

Immunocytochemistry (cell cultures):
The medium was removed and the cells rinsed in Tris buffer (pH = 7.3), fixed in 4% paraformaldehyde for 25 min and rinsed in Tris again. Non-specific staining was blocked in 10% goat serum for 1 hr. Cells were incubated in 1o antisera to TH (Chemicon) or GFAP (Chemicon) overnight at 4oC. After several rinses, cells were incubated in biotin-conjugated goat anti-mouse IgG 2o antibody (Chemicon) for 1 hr, rinsed again, and incubated in ABC-peroxidase reagent (Vector Labs) for 1 hr. Following several rinses, labeling was visualized by exposure to 0.5 mg/ml 3,3' diaminobenzidine (DAB) and 0.03% H2O2 in Tris buffer.

In situ hybridization:
Fresh-frozen, cryostat-cut, slide-mounted sections through the E14 rat mesencephalon were processed for the detection of erbB4 and TH mRNAs by using *in situ* hybridization with 35S-labeled cRNA probes, as described previously (Seroogy & Herman, '97; Numan & Seroogy, '99; Kornblum et al., '00; Zhang et al., '01a). The cDNA template used to produce the ErbB4 cRNA was kindly supplied by H. Kornblum (UCLA). After post-hybridization ribonuclease treatment and washes, the sections were exposed to ß-Max Hyperfilm (Amersham) for 3-6 days to generate film autoradiograms, and then coated with NTB2 nuclear track emulsion (Kodak; 1:1 in H2O) for 3-4 weeks to generate emulsion autoradiograms.

Western blots:
Cells treated with and without GGF2 were harvested on DIV 7 and 14. Protein concentrations of the lysates were measured by using the BCA Protein Assay Kit (Pierce). Solubilized proteins were separated by SDS-PAGE (7.5% gel) and transferred to Hybond membranes. The membrane was blocked in 5% skim milk in TBST buffer for 1 hr, then incubated with 1o antibody (anti-ErbB2, anti-ErbB3, anti-ErbB4; all antibodies from NeoMarkers; anti-GFAP, Chemicon) overnight at 4oC. After washing, the membranes were incubated with HRP-conjugated 2o antibodies (1:2000; Sigma). Subsequently, membrane-bound HRP-labeled antibodies were detected by an enhanced chemiluminescence detection system (ECL-Plus; Amersham) and exposed to Fuji x-ray film. The films were quantified by using Quantity One software (Bio-Rad).

Cell counts:
TH-positive (dopaminergic) and GFAP-positive (glial) cells were counted manually (by a blinded observer) in nine adjacent center microisland fields at 40X magnification, accounting for approximately 25% of the area of the island.

Results

1. ErbB4 mRNA expression in E14 rat ventral mesencephalic flexure overlaps with TH mRNA

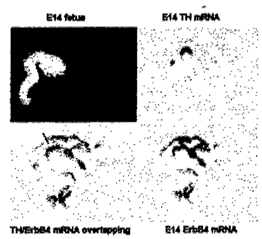

Fig. 1. *In situ* hybridization of TH and ErbB4 mRNAs in E14 rat. Note substantial overlap in expression of both mRNAs in the ventral mesencephalic flexure (arrow).

2. ErbB2, ErbB3 and ErbB4 receptor proteins are expressed in developing rat mesencephalic cultures

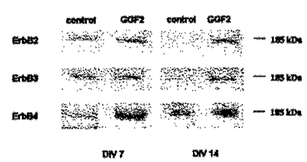

Fig. 2. Western blots were performed on rat midbrain cultures on DIV 7 & DIV 14. The panel shows that ErbB2, ErbB3 and ErbB4 proteins are expressed in both control and GGF2-treated cell cultures.

3. ErbB2, ErbB3 and ErbB4 receptor proteins are up-regulated by GGF2 in midbrain cultures

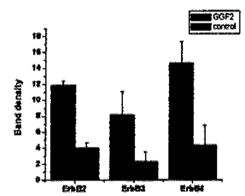

Fig. 3. Densitometric analysis of Western blots from midbrain cultures showing up-regulation of ErbB2, ErbB3 and ErbB4 receptor proteins in GGF2-treated versus control cultures on DIV 14.

4. GGF2 promotes the long-term survival of cultured dopaminergic neurons

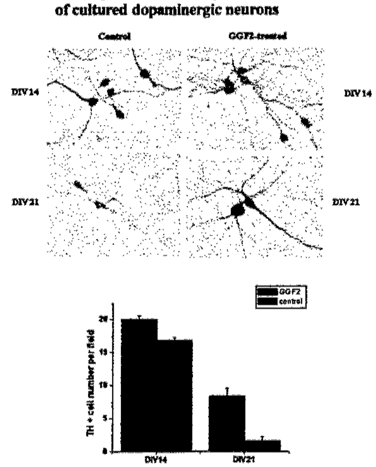

Fig. 4. *(Upper panel)* Immunostaining for TH in DIV 14 & 21 midbrain cultures treated with or without GGF2. More TH+ neurons with longer processes are apparent in the GGF2-treated cultures compared to the control cultures, especially on DIV 21. *(Lower panel)* Cell counts of TH+ cells in control and GGF2-treated cultures on DIV 14 and 21.

5. GGF2 promotes glial proliferation in developing rat mesencephalic cultures

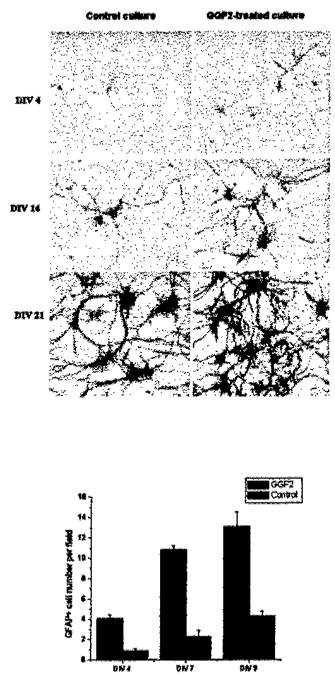

Fig. 5. *(Upper panel)* Immunostaining for GFAP in mesencephalic cultures treated with or without GGF2 on DIV 4, 7 and 14. *(Lower panel)* Cell counts of GFAP+ cells in control and GGF2-treated cultures on DIV 4, 7 and 9. Note substantial increase in the number of GFAP+ cells in GGF2-treated versus control cultures.

6. Western blots for GFAP in developing midbrain cell cultures on DIV 7 and DIV 14

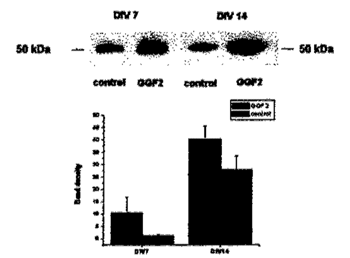

Fig. 6. Western blots for GFAP protein in developing midbrain cell cultures on DIV 7 and 14. GFAP protein expression in GGF2-treated cultures is increased compared to that in control cultures.

Summary & Conclusions

- ErbB4 receptor mRNA is expressed in rat E14 ventral mesencephalic flexure in an overlapping pattern with TH mRNA
- ErbB2, ErbB3 and ErbB4 receptor proteins are expressed in rat primary mesencephalic cell cultures
- GGF2 up-regulates ErbB2, ErbB3 & ErbB4 proteins in developing rat midbrain cultures
- GGF2 promotes the long-term (but not short-term) survival of dopaminergic neurons in rat primary midbrain cultures
- GGF2 promotes mesencephalic glial proliferation *in vitro*
- The survival and neuroprotective effects of GGF2 on dopaminergic neurons in developing midbrain cultures may be mediated by glia

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Topic 1: Cytokines and other factors: expression and biological effects

Abstract Title: THE LONG-TERM SURVIVAL OF CULTURED DOPAMINERGIC NEURONS IS PROMOTED BY THE NEUREGULIN GGF2

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Key words: Survival, Midbrain, ErbB, Parkinson's disease

Abstract: We previously reported that the growth/differentiation factor glial growth factor-2 (GGF2), an isoform derived from the neuregulin-1 gene, protected dopaminergic neurons against serum deprivation and 6-hydroxydopamine-induced degeneration, but did not appear to affect early survival of the developing dopamine cells in fetal rat primary midbrain cultures. The current study was conducted to determine if GGF2 could promote long-term survival of the dopaminergic neurons in vitro. Cells plated at low density were given 5% fetal bovine serum for 48 hours, afterwhich, cells were changed to fresh, serum-free culture medium and treated with either GGF2 (100 ng/ml) or vehicle. Numbers of tyrosine hydroxylase (TH)-immunoreactive (dopaminergic) neurons and glial fibrillary acidic protein (GFAP)- immunoreactive (glial) cells were subsequently assessed at 7, 14, and 21 days in vitro (DIV). The numbers of both TH- and GFAP-positive cells were

significantly increased at DIV 21, compared to controls. Western blot analysis of parallel cultures revealed the expression of the neuregulin receptors ErbB2 and ErbB4, but not ErbB3, in control cultures. However, after GGF2 treatment levels of ErbB2 and ErbB3, but not ErbB4, were up-regulated. These results indicate that GGF2 promotes the long-term survival of dopaminergic neurons and differentially regulates expression of its' receptors in developing midbrain cultures, possibly via glial mechanisms. Supported by NIH grants NS39128 and NS35890 and DOD grant DAMD 17-02-0174.

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